PHOTOPERIODIC CONTROL OF A PHYSIOLOGICAL RHYTHM[1]

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A hormone, proctodone, was recently discovered to be produced by epithelial cells located in the anterior portion of the proctodeum of the larval European corn borer, *Ostrinia nubilalis* (Hübner) (Beck and Alexander, 1964a). Proctodone appeared to be responsible for the activation of the neurosecretory processes leading to the production of the prothoracotropic hormone, thereby constituting a major endocrine component of the physiological processes underlying diapause development and prepupal morphogenesis (Beck and Alexander, 1964b). Cytological evidence of daily secretory cycles in the proctodone-producing epithelial cells was obtained, and some of the possible relationships between rhythmic physiological functions and extrinsic photoperiodic signals have been discussed (Beck, 1964).

In the earlier publications, cited above, it was postulated that the rate of diapause development may be determined by the degree of synchronization between two or more interacting or interdependent physiological rhythms, such that when the rhythms were physiologically "in phase," development was rapid and diapause was soon terminated. Conversely, diapause development was slow and the diapause state greatly prolonged when the participating rhythmic functions were physiologically "out of phase." In support of this "phase theory" of developmental control, evidence was presented of the existence of secretory rhythms in both proctodone-producing epithelium of the proctodeum and lateral neurosecretory cells of the larval brain (Beck, 1964). The rhythms were found to be influenced by the environmental photoperiod, with the neurosecretory rhythm being sensitive to the onset of light and the proctodeal rhythm being sensitive to the onset of darkness. Both rhythms were found to be characterized by the possession of an 8-hour period, rather than the expected 24-hour period. Since the rhythms ran through three full cycles per day, they cannot be classified as circadian rhythms, although they certainly utilized circadian photoperiodic signals as "Zeitgeberen."

The present study was undertaken in an effort to clarify some of the relationships between photoperiodic signals and the temporal characteristics of the proctodeal secretory rhythm. Emphasis has been on the cytological changes involved in the rhythmic function, rather than on the role or fate of the physiologically active substances produced during the secretory process. Although a number of physiological processes, including some endocrine functions, have been shown to display rhythmicity in plants, invertebrates, and vertebrates, there has been a marked paucity of cytological evidence in support of the hypothesis that secretory processes may show rhythmic characteristics (Halberg, 1960; Harker, 1960; Bünning, 1963; Beck, 1963; Wolfson, 1964).

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Methods and Materials

The experimental insects employed in this investigation were larvae of the European corn borer. The larvae were reared on meridic diets as previously described (Beck, 1962), with diapause being induced by means of short-day photoperiods (12 hours of light and 12 hours of dark) during the larval growth period. Some experiments involved exposure of larvae to long-day conditions, which was accomplished by means of incubators programmed for 16 hours of light and 8 hours of dark per day. All of the short-day and long-day incubators were synchronized so that the clock times of the onset of light were identical. The photoperiodic conditions differed, therefore, only in the clock times at which the lights-off signal occurred. With only a few exceptions, noted specifically below, the temperature used for rearing, treatment, and post-treatment was 30° C.

Tissue samples for permanent slide preparation were fixed in Bouin's fixative, imbedded in paraffin, sectioned at 7 microns, and stained. Staining was by either iron hematoxylin and eosin or the paraldehyde-fuchsin technique of Cameron and Steele (1959). Nearly all of the experiments involved the examination of fresh proctodeal tissue for the presence of intracellular secretory granules. This was accomplished by dark-field fluorescence microscopy; a Leitz ultraviolet accessory was used, employing a UG-1 2-mm. exciter filter and Euphos barrier filters. Living larval proctodeums were dissected out, and excess fat and tracheae removed. They were then split laterally, mounted flat in water on a microscope slide, and covered with a thin coverslip.

The secretory granules occurring in the proctodeal epithelium were previously observed to display autofluorescence, leading to the detection of cyclic cell activity (Beck, 1964). In the present study, the secretory cycle was followed by the examination of tissue samples collected at different times during the photoperiodic cycle. Permanent records of the relative intensity of the fluorescence and the cellular disposition of the fluorescing granules were obtained by photomicrography. For this purpose, 35 mm. color film was used (High-Speed Ektachrome, ASA = 160) with a standard shutter speed of 120 seconds. The pictures were developed and mounted as 2×2 inch transparencies. All subsequent considerations of the secretory state of the samples were on the basis of the colored photomicrographs, as the tissue samples themselves could not be preserved.

In order to study the secretory cycle and the effects of variables on the cycle, it was necessary to devise a method for comparing the secretory state of different tissue samples. For this purpose, the secretory cycle was divided into a sequence of 5 arbitrary stages based on the characteristics of the fluorescence recorded in the colored photomicrographs. Black and white prints of the photomicrographs constituting the standard examples of each stage are presented as Figures 1–5. The general distinguishing characteristics of each of the several stages are given with the figures. Although subject to the limitations invariably accompanying an atomistic consideration of a continuum, the use of this arbitrary scale of relative secretory activity has facilitated the study and has yielded consistent and reproducible results.

With only few exceptions, all of the epithelial cells of a given proctodeal sample were virtually identical in respect to the observable fluorescent inclusions, making

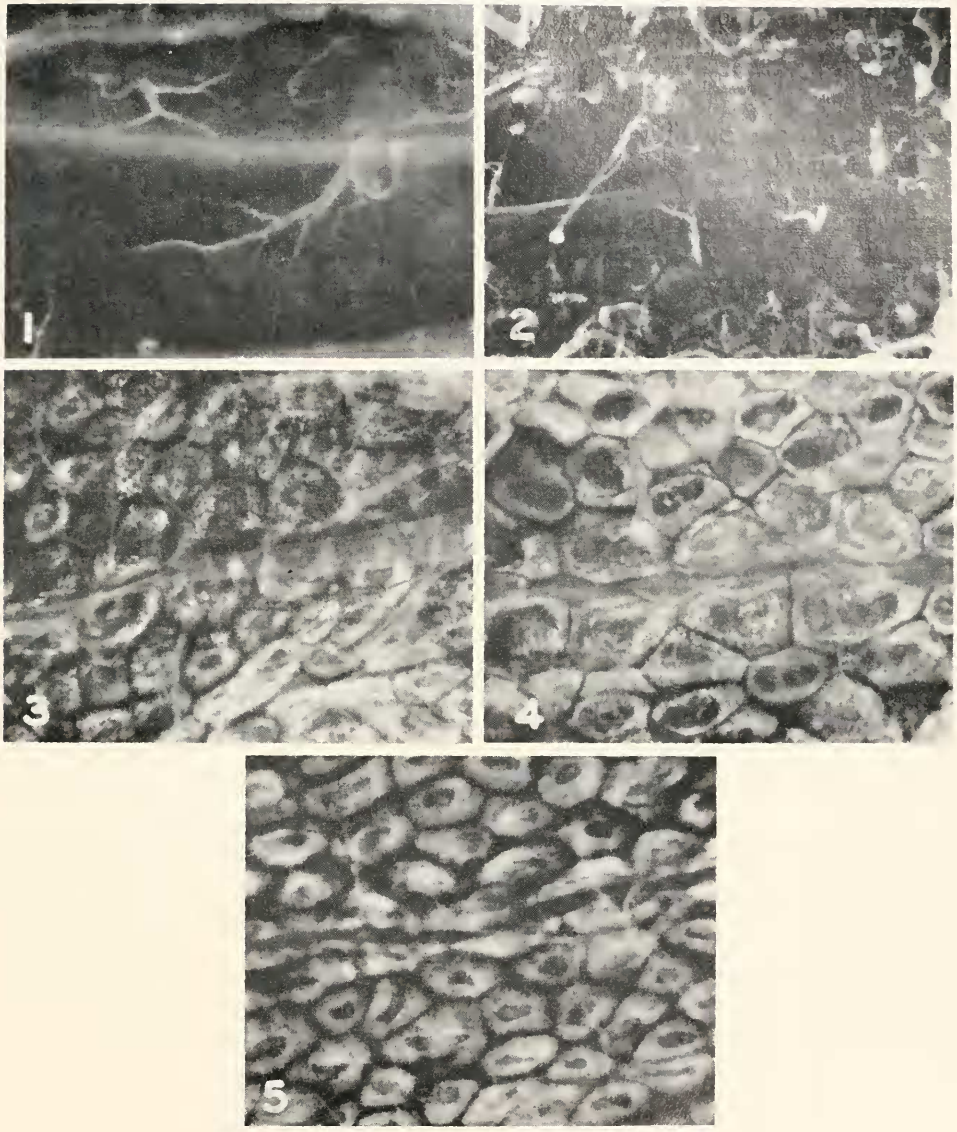

FIGURE 1. Stage 0 of proctodeal secretory cycle. Cells are devoid of visible fluorescent cytoplasmic granules.

FIGURE 2. Stage 1 of proctodeal secretory cycle. Cells contain scattered fluorescent granules; cell outlines are just discernible.

FIGURE 3. Stage 2 of proctodeal secretory cycle. Cells contain numerous large fluorescent granules; cell and nuclear outlines easily discernible.

FIGURE 4. Stage 3 of proctodeal secretory cycle. Fluorescent cytoplasmic granules vary numerous; nuclear outlines well-defined.

FIGURE 5. Stage 4 of proctodeal secretory cycle. Cells packed with finely divided fluorescent inclusions; nuclei partially obscured by inclusions.

it relatively easy to assign each preparation to one or another of the stage classifications. The procedure for assigning secretory stage values to the photomicrographs was as objective as practicable. The pictures obtained in an experiment or series of tissue samples were marked with code numbers, randomized, and compared individually with the series of standards. On the basis of such comparison, each was assigned to one of the arbitrary stages. Following this evaluation, the photomicrographs were decoded, and the stage values were recorded according to the experimental series to which they belonged. Statistical analyses of the data so obtained were limited to frequency distribution considerations. The use of statistical tests, such as analyses of variance, was avoided because of the mathematical artificiality of numerical data describing arbitrary classifications.

Results and Discussion

Rhythm characteristics

Stained sections of the anterior portions of the proctodeums of mature diapausing European corn borer larvae usually disclosed that the epithelial cells contained granular cytoplasmic inclusions that stained dark purple with paraldehyde-fuchsin. The presence of such granules, irregular multilobate nuclei, and numerous small cytoplasmic vacuoles was interpreted as being indicative of much synthetic activity. In some specimens, the cells were devoid of the stainable granules, although showing the typical vacuoles and multilobate nuclei. Whether or not the epithelial cells contained stainable secretory granules appeared to depend upon the time of day that the larvae had been fixed. These observations suggested that secretory activity was on a cyclic basis. Our interpretation has been that the paraldehyde-positive products accumulate in the small cytoplasmic vacuoles, and are then secreted into the hemolymph through the basal cytoplasmic membrane and the basement membrane underlying the cell layer. In the immature, feeding stages of the insect, these cells are known to absorb water and digested substances from the gut lumen and to secrete them into the hemolymph (Wigglesworth, 1950). During diapause and postdiapause, the larvae do not feed and the gut tract is quite empty. Our observations indicate, however, that the cells retain the capability of secretion into the hemolymph, and have an endocrine function.

Study of the secretory cycle has been greatly facilitated by the finding that the secretory granules display a pale green autofluorescence in the fresh state. The cycle of elaboration and secretion could be followed by the examination of successive timed tissue samples without the laborious delay involved in fixation, embedding, sectioning, and staining. It was found that at the stage of the secretory cycle in which no paraldehyde-fuchsin-staining granules were present, no fluorescent granules were detectable. This observation led us to the tentative interpretation that the stainable particles and fluorescent particles were identical.

The absence of fluorescent inclusions in the epithelial cells was characterized as Stage 0 of the secretory cycle (Fig. 1), and was interpreted as being the result of the cells' having liberated their accumulated secretory products. For reasons to be discussed below, this stage of the secretory cycle is thought to be of relatively short duration. After Stage 0, the cells again begin to accumulate secretory products, as shown by progressive increase in the amount of fluorescent granules—

Stages 1, 2, 3, and 4 (Figs. 2–5). Our observations have led us to believe that the cells are capable of liberating their products after they reach either Stage 3 or 4. For the purposes of studying the effects of photoperiod on the secretory cycle, Stages 0 and 1 were of the greatest interest, and their occurrence was contrasted to the occurrence of the other secretory stages.

Proctodeal tissue samples were taken hourly from groups of diapausing borer larvae that were maintained under either long-day or short-day photoperiods. The results of typical 24-hour series are shown in Figures 6 and 7, in which each tissue sample examined is symbolized by a small circle, with the occurrence of Stages 0 and 1 being depicted by solid black circles and the other stages by open circles.

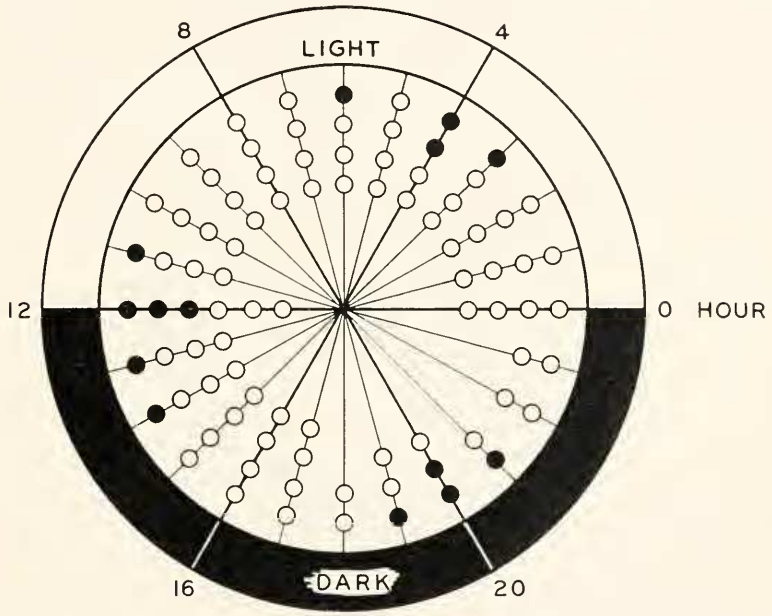

FIGURE 6. Temporal distribution of proctodeal secretory cycle Stages 0 and 1 (solid circles) among diapausing larvae of the European corn borer held in a short-day photoperiod.

It was apparent that Stages 0 and 1 did not occur at random during the 24 hours, but tended to be most frequent at the beginning of the scotophase (dark phase of the photoperiod) and at 8-hour intervals thereafter. The borer larvae used in the series depicted in Figure 7 had been transferred from a short-day photoperiod to a long-day photoperiod 10 days prior to the experiment. The results indicate, therefore, that the insects were capable of adjusting the secretory cycle in accord with the photoperiod and that the beginning of the scotophase determines the time of occurrence of the subsequent secretion times. We have confirmed this conclusion by the results of a number of experiments in which the borer larvae were required to adjust to changes in the time of the beginning of the scotophase; the secretory rhythm always shifted so that one of the times of secretion (Stages 0 and 1) approximately coincided with the onset of the scotophase. The proctodeal

secretory rhythm is temporally adjusted through the insect's response to the lights-off stimulus provided by the extrinsic photoperiod.

The proctodeal secretory rhythm is not, in itself, a circadian rhythm, because it displays a period approximating 8 rather than 24 hours. It is phase-set (temporally adjusted) by the 24-hour rhythm of daylight and darkness, and is most certainly a component of the insect's photoperiodism. The possible significance of the effects of lights-on and lights-off stimuli on 8-hour physiological rhythms in the control of growth processes was discussed in an earlier paper (Beck, 1964).

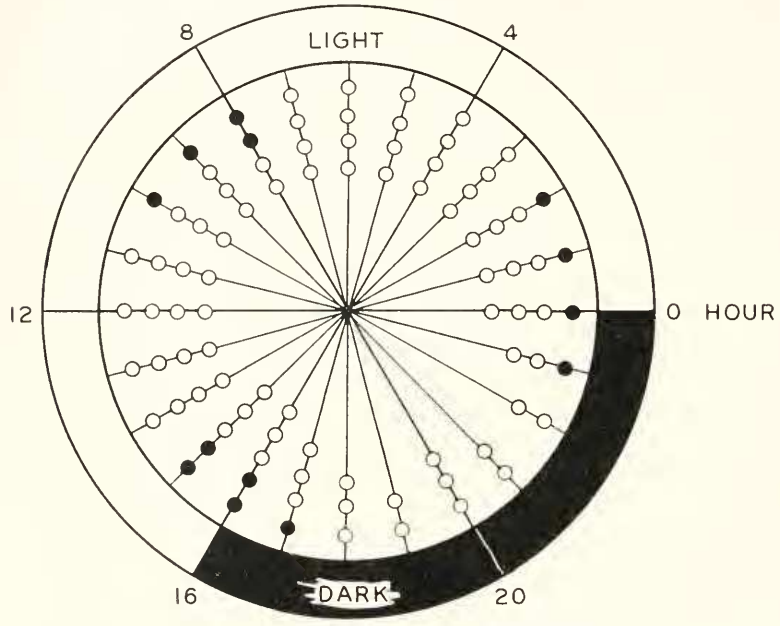

FIGURE 7. Temporal distribution of proctodeal secretory cycle Stages 0 and 1 (solid circles) among diapausing larvae of the European corn borer held in a long-day photoperiod.

Data such as those of Figures 6 and 7 gave rise to some serious questions concerning the actual time-course of the physiological rhythm. One such question pertained to an explanation of the relatively low frequency of occurrence of Stages 0 and 1 at the beginning of the scotophase. Not all (or even most) of the larvae sacrificed at that time showed proctodeal tissue that was nearly devoid of fluorescent granules. One might expect that the incidence of Stages 0 and 1 would be much higher than was actually observed, if the population was to be considered reasonably homogeneous and if all of the larvae were responsive to the photoperiodic stimuli. These considerations prompted us to undertake a more detailed study of the distribution of secretory stages during the hours immediately before and after the beginning of the scotophase.

The percentage incidence of the several arbitrary stages of secretion for some different time periods is shown in Figure 8. "Midcycle" is the 60-minute period between 4.5 and 3.5 hours prior to the lights-off time; "−1 hr." identifies samples

examined between 90 and 30 minutes prior to the lights-off time; "0 hr." designates samples taken from 30 minutes before to 30 minutes after the lights-off time; and "+1 hr." designates tissue samples collected from 30 to 90 minutes after the beginning of the scotophase. About 40 larvae were dissected and examined to obtain the data for each of the different time periods.

The "midcycle" distribution shows Stage 3 to be the most frequent, with Stages 3 and 4 constituting the condition of over 80% of the insects examined. At this time there were no Stage 0, and Stage 1 was rare (one instance out of a total sample of 40 larvae). At "−1 hr." the distribution changed only in that Stage 0 and

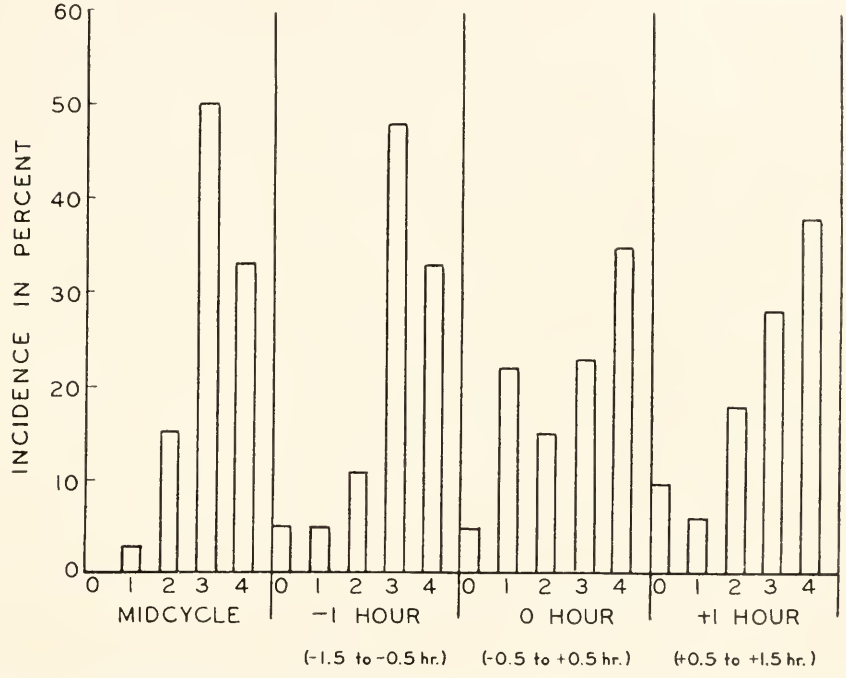

FIGURE 8. Incidence of different arbitrary proctodeal secretory stages (0–4) among European corn borer larvae examined at different times during the secretory cycle.

Stage 1 were each present to the extent of about 5%. The distribution at "0 hour" showed two pronounced changes: (a) the incidence of Stage 1 was greatly increased, and (b) the incidence of Stage 3 was much lower than in previous samplings. The distribution at "+1 hour" showed a decline in the incidence of Stage 1 and an increase in Stage 3. Although not included in Figure 8, histograms of the incidence of the different secretory stages at +2 and +3 hours showed a return to the "midcycle" distribution.

The cell state observed in Stage 0 and Stage 1 proctodeal tissue samples was interpreted as an indication that the cells had liberated their secretory products shortly before the larvae had been dissected. For this reason, the incidences of these two secretory stages were considered together, and their incidence compared

to the incidence of the other arbitrary stages in subsequent experiments. Combining the data from a number of series in which the larvae examined were from the standard short-day photoperiod, the temporal distribution characteristics of Stages 0 and 1 were determined for the period from two hours before to two hours after the beginning of the scotophase (Fig. 9). Of the total number of Stages 0 and 1 observed, 43% occurred within 30 minutes of the beginning of darkness. The data form an obviously normal frequency distribution, with the mean falling very close to the lights-off time of the photoperiodic cycle. From these data, we can conclude that the insect's proctodeal secretory rhythm is, indeed, temporally adjusted to

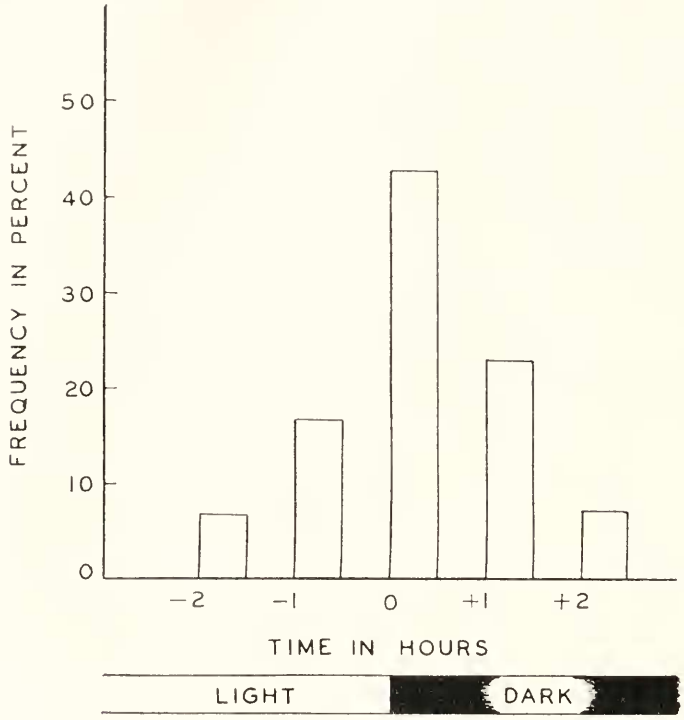

FIGURE 9. Frequency distribution of combined Stages 0 and 1 during the hours surrounding the beginning of the scotophase.

the beginning of the scotophase, such that the proctodeal cells tend to release their secretory products in approximate synchrony with the lights-off signal. Because the secretory rhythm appears to possess an eight-hour period, it is evident that photoperiod can directly influence only one of the three daily cycles; the others must occur endogenously, but timed in accord with the cycle that responded to the photoperiod.

Sampling probability characteristics

If the proctodeal cells liberate their secretory products in approximate synchrony with the lights-off signal, why is the incidence of Stages 0 and 1 often relatively

low, even at the "0 hour" as shown in Figure 8? This effect can be explained on the basis of the unfavorable probabilities attending the tissue sampling procedure. The measured response (Stages 0 and 1) is assumed to form a normal distribution such that two-thirds of the larvae may be expected to respond within 60 minutes on either side of the lights-off time. A second assumption is also made: the duration of Stage 0 is about 15 minutes and that of Stage 1 is about 30 minutes. When a larva is chosen at random for dissection at about the beginning of the scotophase, the probability that it will be among those that respond within the two-hour

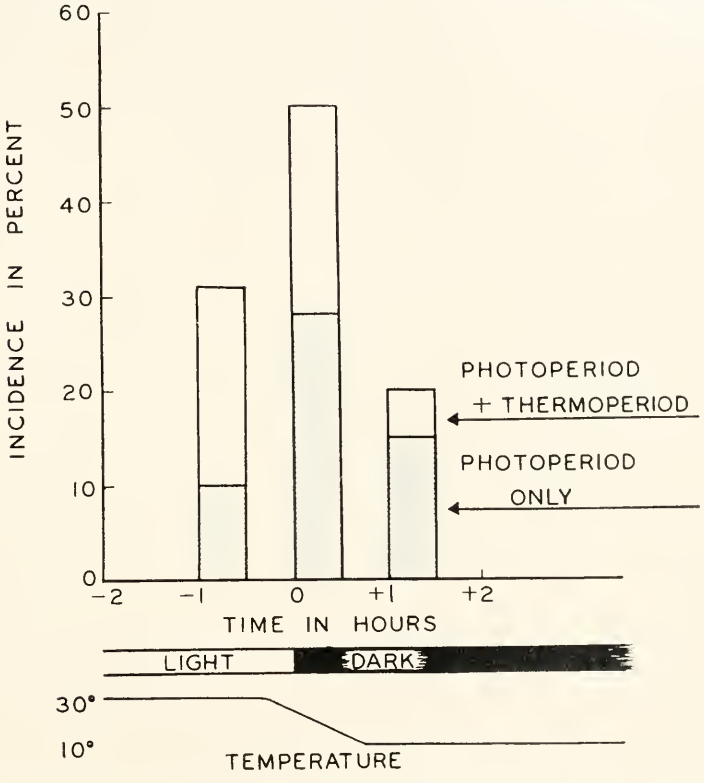

FIGURE 10. Effect of a combined thermoperiod and photoperiod on the incidence of Stages 0 and 1 of the proctodeal secretory cycle in diapausing larvae of the European corn borer.

period designated above is 0.67. The probability that it will be at Stage 0 at the time of dissection is 0.67×0.125 (because there are eight 15-minute periods in two hours). This means that the probability of observing a Stage 0 tissue preparation at even so favorable a time is but 0.083; on the average, one larva out of a sample of twelve should be found to be at Stage 0. If the duration of Stage 1 is twice that of Stage 0, about one larva out of six should be found to be at this stage when the population is sampled during the hour before and the hour after the lights-off time. The expected incidence of Stages 0 and 1 combined would be about 25% of the larvae examined; the "0 hour" distribution data in Figure 8 show a combined Stages 0 and 1 incidence of 28%.

These probability considerations have some important implications that reach beyond the present limited study. In any instance where a physiological process cannot be followed continuously in each individual specimen, the investigator is limited to making observations on a succession of specimens, each of which has been fixed at some point of time. Under such conditions, especially those in which successive physiological stages are relatively fleeting, the existence of highly significant rhythmic functions may be overlooked or, if suspected, difficult to demonstrate convincingly. In the present investigation, the demonstration of a photoperiodically sensitive secretory rhythm could not have been accomplished in the absence of the moderately large numbers of samples made feasible by the simple autofluorescence technique of examination.

Effects of thermoperiod

An attempt was made to increase the incidence of Stages 0 and 1 among the borer larvae at the time of the lights-off signal. A thermoperiod was superimposed on the photoperiod, so that the incubator temperature fell from 30° C. during the photophase to 10° C. during the scotophase. Previous results (Beck, 1962) had shown that low temperatures during the scotophase increased the incidence of diapause, whereas high temperatures at that time tended to prevent diapause. As shown in Figure 10, combining a thermoperiod with the short-day photoperiod had the expected result of greatly increasing the incidence of observed Stages 0 and 1. The large increase in response that occurred at one hour before the light-off signal indicated that the effect was not a simple prolongation of the duration of Stages 0 and 1 because of low ambient temperature. It seems more likely that the effect of the thermoperiod was one of decreasing the time range of the response, and thereby improving the probability of observing specimens in Stages 0 and 1 during the two-hour period of interest.

Short-day to long-day transition

That the secretory rhythm was capable of adjusting to a changed photoperiodic schedule within a 10-day period was apparent early in the study (Figure 6 compared to Figure 7). Attempts to trace the detailed course of such a transition have not been wholly successful, however, because of the limitations of our sampling methods and the inherent variability among the individuals of the experimental populations used.

A large group of borer larvae that had been reared under a short-day schedule in which the lights-off signal came at 12 noon was transferred to a long-day schedule in which the lights-off signal came at 4 PM. The incidence of Stages 0 and 1 was determined for three time periods: (a) 11 AM to 1 PM, which was one hour on each side of the lights-off time of the original short-day photoperiod; (b) 1:30 to 2:30 PM, which was midway between the old and new scheduled lights-off time; and (c) 3 to 5 PM, which was one hour on each side of the lights-off signal of the newly imposed photoperiod. The data of Figure 11 are typical of the results obtained from experiments of this type. The incidence of larvae responding in accord with the original noontime lights-off signal declined during the 8 days of the experiment. Responses during the 1:30 to 2:30 interval were observed throughout

the 8 days, with the incidence about constant from the second day on. As expected, no larvae responded between 3 and 5 PM on day 0, but the incidence of response increased from day 1 until at least day 6. The results indicate that there was considerable individual variation in the larvae's ability to adjust to a four-hour change in the photoperiod. Some larvae were responsive to the new photoperiod by the second day, whereas others were still in the process of making the transition on the 8th day, as evidenced by the incidence of responses occurring in the middle hour of 1:30 to 2:30 PM. Although it was apparent that transient phases occurred during the process of becoming synchronized with a changed photoperiod, we were unable to determine the number of transient responses involved. Nonetheless, these results are in very good agreement with those of earlier work (McLeod and Beck, 1963), in which the effect of photoperiod on diapause development was

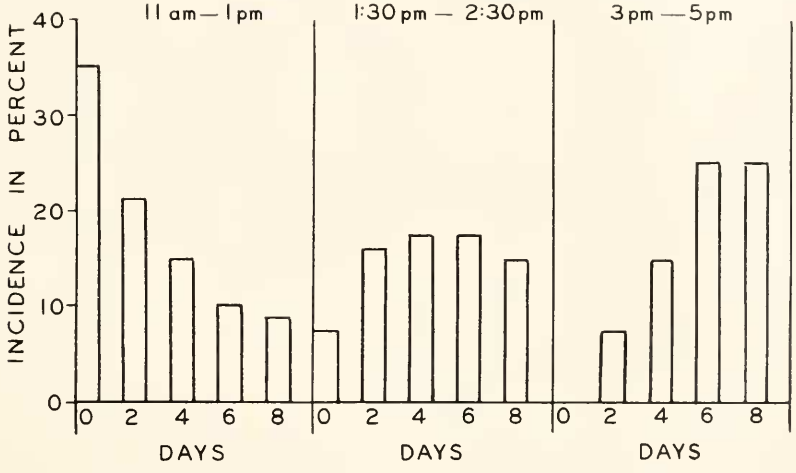

FIGURE 11. Incidence of secretory Stages 0 and 1 during 8 days following a four-hour change in the time of the lights-off signal (European corn borer larvae in diapause).

studied. In that work, transfer of diapausing borer larvae from short-day to long-day photoperiods and from long-days into continuous darkness disclosed that some of the larvae could adopt and maintain a long-day developmental schedule within two days, but at least 10 days were required for all of the population to make the adjustment.

SUMMARY

1. In mature diapausing larvae of the European corn borer, *Ostrinia nubilalis* (Hübner), the epithelial cells of the anterior portion of the proctodeum were observed to show evidence of a regular cycle of synthesis and secretion. The cycle involved progressive accumulation and subsequent disappearance of paraldehyde-positive, autofluorescent cytoplasmic inclusions. For the purposes of this study, the secretory cycle was divided into a series of 5 arbitrary secretory stages.

2. The proctodeal secretory activity was shown to constitute a rhythmic function. The rhythm was found to be non-circadian, in that it displayed an approxi-

mately 8-hour period, such that three complete secretory cycles occurred during each 24-hour day.

3. The proctodeal secretory rhythm was shown to be phase-set by photoperiod. The release of secretions (loss of intracellular granules) occurred in response to the beginning of the scotophase (lights-off stimulus) and endogenously every 8 hours thereafter.

4. A thermoperiod that was superimposed on the photoperiod, so that a low temperature was concurrent with the scotophase, had the effect of intensifying the physiological response to the photoperiod.

5. From two to ten days were required for the secretory rhythm to re-synchronize with a four-hour change in the clock time of the lights-off signal. Because of great individual variability, the number of transient phase responses could not be determined.

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